The impact of microRNA-3151 in *BRAF*-mutated human malignancies

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation
with honors research distinction in Molecular Genetics in the
undergraduate colleges of The Ohio State University

by

Malori A. Lankenau

The Ohio State University
May 2015

Project Advisor: Professor Albert de la Chapelle, Department of Molecular Genetics

Acknowledgements

I would like to especially thank my mentors, Dr. Albert de la Chapelle and Dr. Ann-Kathrin Eisfeld, as none of this work could have been done without their support and guidance. I am grateful to them for letting me join the lab and work on this project and I appreciate their patience and encouragement as I learned the experimental techniques. The knowledge I have gained over the past two years working in the lab under Dr. de la Chapelle and Dr. Eisfeld has been essential to not only the completion of this thesis, but to my overall education and undergraduate degree. I want to also thank Ravi Patel, Kevin Hoag, and Dr. Christopher Walker for their help and assistance in completing the experiments and Sandya Liyanarachchi for the statistical analysis. I would like to give a special thanks to our lab manager, Jan Lockman, who has supported and assisted me since I began in the lab. She is essential to keeping the lab running, and has personally helped me with completing this thesis. I am truly grateful to everyone who has made this possible.

Table of Contents

I. Abstract....4

II. Introduction....5

III. Statement of Significance....11

IV. Results....12

V. Discussion....29

VI. Materials and Methods....31

VII. References....35

List of Figures and Tables

Figure 1: Survival rate of melanoma cases based on stage....6

Figure 2: Survival rates of thyroid cancer cases based on stage....7

Figure 3: The TP53 pathway.... 8

Figure 4: Gene expression of target gene with and without the microRNA....9

Figure 5: miR-3151 in MM....13

Figure 6: Direct and indirect targets of miR-3151.... 15

Table 1: Potential direct target genes of miR-3151....15- 16

Table 2: Molecular and Cellular Functions predicted to be influenced by miR-3151 (Ingenuity)....17

Figure 7: Cellular localization of TP53....18

Figure 8: Proliferation rates in response to miR-3151.... 19

Figure 9: Changes in the cell cycle.... 20

Figure 10: Caspase-3 cellular localization.... 21

Figure 11: Effects of BRAFmut on miR-3151 and TP53.... 22

Figure 12: SP1/ NF-κB transactivating complex activates miR-3151 expression....23

Figure 13: miR-3151 in PTC....24

Figure 14: miR-3151's effects on TP53 and Caspase-3 in PTC.... 25

Figure 15: miR-3151 effects on cell viability in PTC....26

Figure 16: BRAFmut effects miR-3151 in PTC.... 26

Figure 17: SP1/ NF-κB activation of miR-3151 in PTC....26

Figure 18: Schematic depiction of the proposed BRAF-miR-3151-TP53 axis.... 27

Figure 19: Vemurafenib treatment of MM and PTC cells.... 28

Figure 20: Images after vemurafenib treatment.... 29

Figure 21: Effect of vemurafenib resistance on miR-3151.... 29

Abstract

The gene *B-Raf proto-oncogene serine/threonine kinase (BRAF)* is the most frequently mutated gene in malignant melanoma (MM) and is causally involved in melanoma and papillary thyroid cancer (PTC) carcinogenesis. Mutated *BRAF* is associated with an aggressive disease phenotype, thus making it a top candidate for targeted treatment strategies in MM and PTC patients. We show that mutations in *BRAF* in both MM and PTC lead to increased expression of a small non-coding RNA, miR-3151. Reducing miR-3151 expression decreases cell viability and increases apoptosis rates in MM and PTC. Thus, miR-3151 may be a novel downstream effector of mutated BRAF, which contributes to the aggressive phenotype associated with BRAF mutations. Using a targeted RNA sequencing approach we determined that miR-3151 directly targets TP53 and other members of the TP53 pathway. As determined by confocal microscopy and nuclear fractionation, knock-down of miR-3151 in MM and PTC cells increased TP53 expression and also favored its nuclear localization. To determine whether targeted knock-down of miR-3151 may have therapeutic potential in MM and PTC, we tested whether a simultaneous treatment of MM and PTC cells with the FDA-approved BRAF inhibiting drug vemurafenib and knock-down of miR-3151 with a small hairpin RNA would add on to the effects of sole BRAF inhibition. Indeed, combined inhibition of mutated BRAF and miR-3151 significantly increased cell death and lowered the dose of vemurafenib required to achieve the observed effects. Taken together, we suggest miR-3151 as a novel player in BRAF mutated malignancies, which acts by direct targeting of the tumor suppressor TP53 and several of its pathway members. Targeted knock-down of miR-3151 may increase the effectiveness of sole BRAF inhibition in MM and PTC.

Introduction

Malignant Melanoma

The incidence rate of malignant melanoma (MM), a cancer of melanocytes, has dramatically increased within the last decades, with an estimated 76,100 new cases reported in 2014[1]. Melanoma is the fifth most common type of cancer in the U.S[1]. Malignant melanoma occurs when damaged DNA generates oncogenic mutations within the skin cells. This damage is most often caused by exposure to ultraviolet radiation from the sun or tanning beds[2]. Malignant melanomas can be characterized into five basic stages, based on localization and invasiveness. The survival rates of melanoma depend on the stage of the cancer (Figure 1). Melanomas that are restricted to their primary sites, or localized, have the highest rates of survival, while melanomas that have spread to nearby lymph nodes are deemed regional[1]. Metastatic melanomas are *distant* and have the lowest rates of survival[1]. Despite extensive research, metastatic malignant melanomas have one of the poorest survival rates of all human cancers.

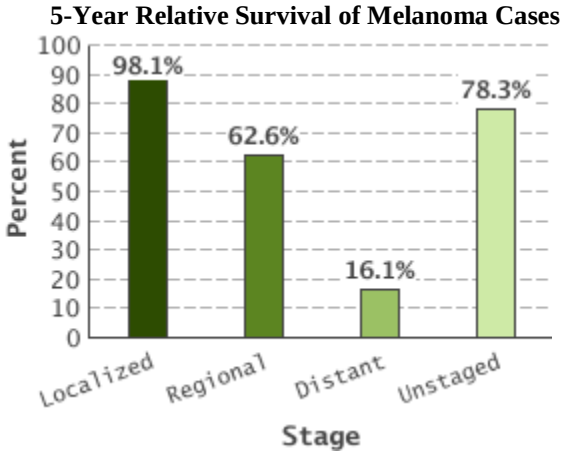


Figure 1. ***Survival rate of melanoma cases based on stage.*** When the cancer becomes metastatic (distant), the survival rate decreases dramatically.

From "SEER Stat Fact Sheets: Melanoma of the Skin"[1].

Papillary Thyroid Cancer

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, accounting for 80% of all thyroid cancers[3]. Incidence rates of PTC are rapidly growing, with over 20,000 new cases

reported each year[3]. Predominantly, cases of PTC occur with no apparent risk factors, although radiation exposure and a family history of PTC can predispose an individual[3]. Fortunately, PTC is the type of thyroid cancer with the highest survival rate and, in contrast to MM, prognosis does not deteriorate as severely with metastasis and invasion. Localized and regional PTCs have comparable rates of survival, and distant PTCs have a lower, but still favorable, prognosis[3] (Figure 2).

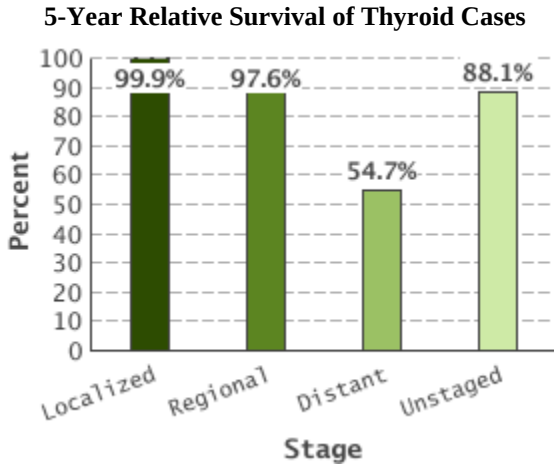


Figure 2. ***Survival rates of thyroid cancer cases based on stage.*** Survival rates of PTC are high when localized and regional. When the cancer becomes distant, prognosis remains favorable.

From "SEER Stat Fact Sheets: Thyroid Cancer"[3].

B-Raf proto-oncogene serine/threonine kinase (BRAF)

Despite the stark differences between MM and PTC in their disease aggressiveness, treatment strategies, and prognosis, there is at least one molecular feature that is shared by both malignancies – frequent mutations in the oncogenic tyrosine kinase *B-Raf proto-oncogene serine/threonine kinase (BRAF). BRAF* is part of the RAS/MAPK pathway, which is involved in many important cellular functions, including proliferation, differentiation, migration, and apoptosis[4]. BRAF is most frequently mutated to a constitutively active form (V600E) in many cancers, including MM (~60%) and PTC (~44%)[1, 5]. When mutated, BRAF is associated with a more aggressive disease phenotype in MM and PTC[2, 7]. Direct inhibition of aberrantly activated

BRAF (e.g, with an FDA approved drug, vemurafenib) has shown some effectiveness, but only in a subset of patients and with the eventual development of resistance in most cases[8].

Tumor protein p53 (TP53)

Additionally, the inactivation of the tumor suppressor gene *tumor protein p53* (*TP53*) is a common event in many MMs[9], as well as a multitude of other human cancers. TP53 is an essential tumor suppressor through its regulation of cell proliferation and apoptosis. Upon cellular stress, TP53 activates one of two pathways, either promoting DNA repair to continue on to the cell cycle or signaling apoptosis (Figure 3). Pharmacologic restoration of *TP53* activity, in combination with BRAF inhibition, represents a promising treatment strategy in MM, and will likely have a potential for a much broader spectrum of patients once agents with higher effectiveness have been identified[10, 11]. Therefore, a better understanding of the deregulated downstream signaling of BRAF and the inactivation of *TP53* are major challenges in MM research.

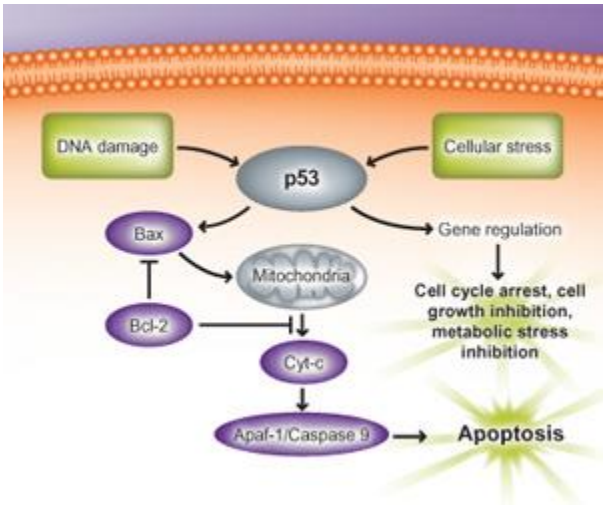


Figure 3. ***The TP53 pathway.*** In response to cellular stress or DNA damage, TP53 activates either a pathway for cell cycle arrest and DNA repair or apoptosis.

From "Apoptosis signaling pathway - @ a glance", *Abcam.com*[43].

Although inactivating mutations in TP53 are found only in 10% of thyroid cancer cases, they are mainly found in poorly differentiated and aggressive tumors[12]. Poorly differentiated tumors lack

normal tissue structures and are comprised of cells with abnormal morphology that poorly resemble the tissue of origin[13]. These tumors typically grow faster and are more likely to spread than well-differentiated tumors[13]. Thus, reviving *TP53* expression could be a possible treatment strategy for this small subset of particularly aggressive tumors.

MicroRNAs

MicroRNAs (miRs) are small, non-coding RNAs, normally 19-26 nucleotides in length, that downregulate the expression of their target gene[14]. miRs downregulate their target genes by the binding of the seed sequence of the miR to the complementary sequence in the 3' UTR of the target gene (Figure 4). miRs downregulate expression through a variety of mechanisms, including inhibiting translation and promoting mRNA degradation. Their target genes vary in different cell and tissue types and potentially regulate a specific pathway through simultaneous regulation of multiple genes. miRs have been associated not only in normal biological process, such as cell proliferation, differentiation, and survival, but also in human disease[15, 16, 17, 18]. Deregulation of specific miRs has been implicated in the disease initiation and progression of virtually every human cancer, including MM[14].

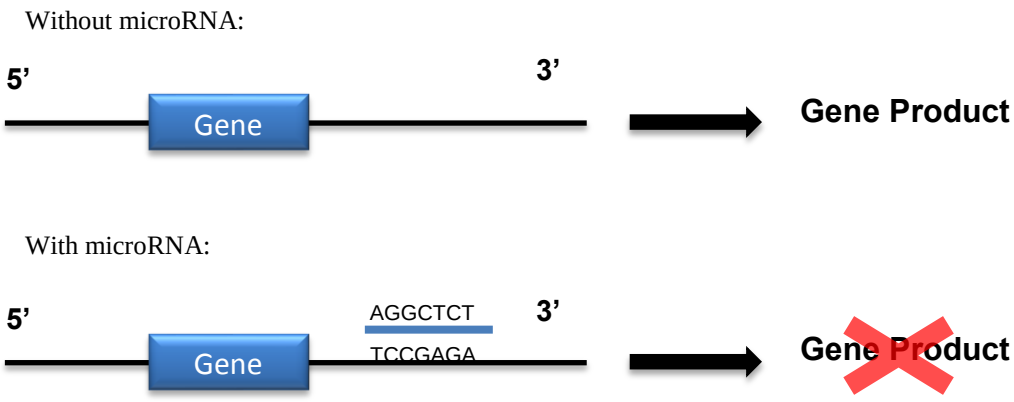


Figure 4: ***Gene expression of target gene with and without the microRNA.*** microRNAs bind to their complementary sequence in the 3' UTR of the target gene to downregulate gene expression via various mechanisms.

Intronic miRs

Although microRNAs can be found throughout the genome, approximately one third of mammalian miRs are located within the introns of their host genes[19, 20]. While some miRs are thought to be co-expressed from the same precursor mRNA in which they reside[21, 22], about 26% of intronic miRs possess their own promoters and can be transcribed as independent units[23, 24, 25]. However, the functional relationship between miRs and their hosts is still largely unknown. Many intronic miRs are believed to strengthen the function of their host genes, either directly by targeting genes within the same cellular pathway[26, 27] or indirectly through silencing genes antagonistic to their hosts[28]. Furthermore, miRs could be shown to be responsible for effects originally contributed to their host genes, and these effects can be attained through expression independent of the host gene[29].

microRNA-3151

Recently, intronic microRNA miR-3151 was identified in intron 1 of the gene *brain and acute leukemia, cytoplasmic (BAALC)* via deep-sequencing of MM samples. In acute myeloid leukemia (AML), high expression of miR-3151 is associated with poor survival of patients and displays direct leukemogenic activity via deregulation of TP53 and several TP53 pathway members[30, 31]. First evidence exists that miR-3151 can downregulate TP53 and decrease apoptosis in MM as well[30]. Additionally, the expression of miR-3151 was found to be deregulated in many MM patients, with BRAF mutated (BRAFmut) tumors having a 5-fold higher expression of miR-3151 when compared to BRAF wild-type (BRAFwt) tumors[30]. Thus, we hypothesized that further elucidation of miR-3151's role in MM, and specifically, its connection with BRAF mutations and TP53, may provide novel insights into melanogenesis, increase our understanding of the BRAF mutant downstream biology, and enhance our options for therapeutic interventions. Furthermore,

we hypothesized that the novel link between BRAF mutations, miR-3151 and TP53 may also apply to other BRAF mutated malignancies such PTC.

Here we tested the above hypothesis by studying the effects of miR-3151 and its knock-down in MM and PTC cells treated or untreated with vemurafenib, a drug used in the therapy of patients with BRAF-mutated MM. We investigated the BRAF-dependent and –independent regulatory mechanisms of miR-3151. We identified the TP53 pathway as a target of miR-3151 and speculate that the combined use of *BRAF*mut inhibitors and knocking down miR-3151 may become a treatment option in patients with BRAF-mutated tumors.

Statement of significance

MM has one of the poorest survival rates of all human malignancies, despite extensive research. BRAF mutations are the most common genetic events in MM and PTC, and are associated with a more aggressive disease phenotype. Additionally, low expression of TP53 is seen in most cases of MM, and is considered a key element of the disease. miR-3151 was identified as a novel player in MM and PTC pathogenesis and is driven by BRAF-dependent and -independent mechanisms. Characterization of TP53 as a downstream effector of miR-3151 provides first evidence for a causal link between *BRAF* mutations and TP53 inactivation. Based on our results, we propose to use the combined inhibition of mutated BRAF and miR-3151 as a target in *BRAF* mutated human malignancies.

Results

AntagomiR-3151 activates the TP53 pathway in malignant melanoma and increases the nuclear localization of TP53.

When assessing miR-3151 in a broader cohort of human cancer samples we recently discovered that miR-3151 levels were 5-fold higher in melanoma primary tumors with an activating mutation in the *BRAF* kinase gene compared to tumors with wild-type *BRAF*[30]. Pilot experiments gave first evidence of TP53 as a possible downstream target of miR-3151 in MM cells[30], which, of note, is inactivated in the majority of MM tumors. These results open the opportunity to tie the presence of *BRAF* mutations to the known low *TP53* expression levels of melanoma cells via miR-3151.

To strengthen the evidence that miR-3151 is an important regulator of *TP53* expression in MM *in vivo*, we determined miR-3151 and *TP53* expression in the tumors of a cohort of MM patients (n=21) and assessed a possible association of the expression levels of the two genes. Patients with high expression of miR-3151 tended to have lower expression of *TP53* and *vice versa* (Figure 5).

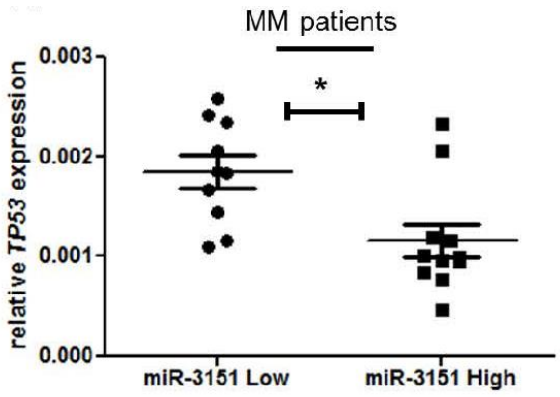


Figure 5. *miR-3151 in MM.* TP53 expression in high miR-3151 and low miR-3151 expressing MM patients, *, $P<0.05$, two-tailed *t* test.

Next, to explore miR-3151's effects on *TP53* and its pathway members in MM, we took a broad

screening approach for the identification of possible target genes. Using the TruSeq RNA platform (Illumina), we custom designed a panel of 361 genes and tested the effects of forced miR-3151 expression and miR-3151 knock-down on the mRNA levels of the panel genes in Mel-39 MM cells. A total of 34 genes showed ≥20% downregulation of their mRNA expression by miR-3151 and concordant ≥20% upregulation by antagomiR-3151 compared to scramble. Therefore these genes were considered as potential direct miR-3151 targets (Figure 6). In addition, 24 genes showed concordant ≥20% upregulation by miR-3151 and downregulation by antagomiR-3151 (Figure 6), suggesting indirect regulatory mechanisms.

As expected, TP53 was downregulated by forced miR-3151 expression and upregulated by antagomiR-3151 (Figure 6). In addition, several of its pathway members were also downregulated, including *BCL2*, *RBL2*, *CASP1*, *CASP9*, *MAP3K4*, and *CDK1* (Figure 6). Eleven of the 34 downregulated genes were predicted to harbor at least 1 binding site for miR-3151 in their 3′-UTR and therefore may be potential direct miR-3151 targets (Table 1).

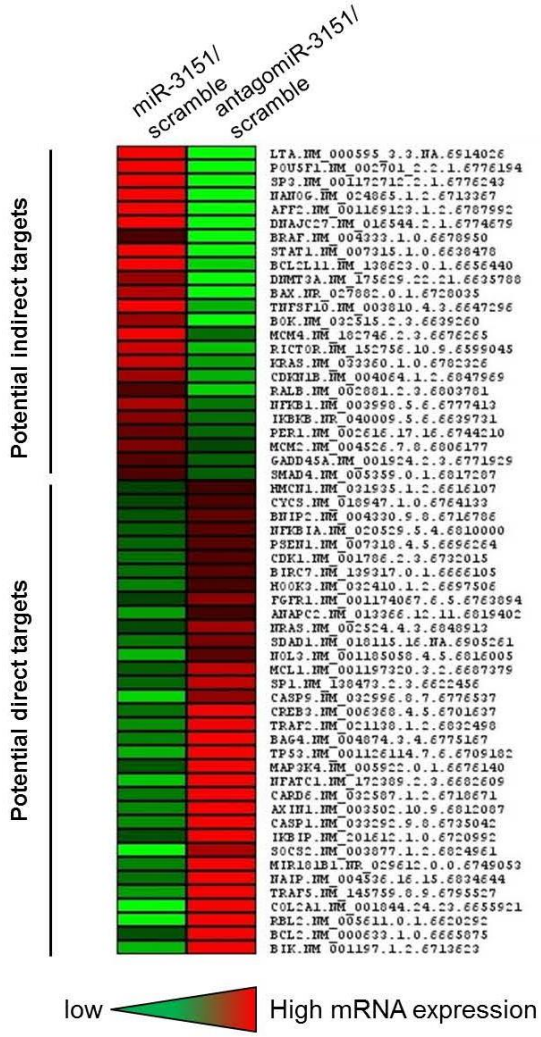


Figure 6. ***Direct and indirect targets of miR-3151.*** Heatmap of gene expression changes concordantly affected by forced miR-3151 expression or miR-3151 knock-down based on targeted RNA sequencing.

Table 1. ***Potential direct target genes of miR-3151.*** Genes which showed concordant ≥20% downregulation in their mRNA expression by miR-3151 and ≥20% upregulation by antagomiR-3151 in the RNA sequencing approach were considered potential direct miR-3151 targets. Their 3′-UTRs were tested for predicted miR-3151 binding sites using a computational prediction approach (microRNA.org). Listed are the genes with predicted miR-3151 binding sites including their affinity scores (miRSVR score and PhastCons score).

Gene	Number of predicted binding sites	mirSVR score	PhastCons score
BIK	1	-0.1048	0.5207
CARD6	1	-0.0107	0.6
NFATC1	1	-0.0006	0.3967
TP53	2	-0.003	0.648
		-0.0063	0.4148
SP1	2	-0.0041	0.653
		-0.0022	0.6501
MCL1	2	-0.0011	0.4804
		-0.2373	0.6276
NOL3	1	-0.0093	0.5414
NRAS	2	-0.0023	0.5623
		-0.0036	0.5216
ANAPC2	1	-0.0026	0.475
FGFR1	1	-0.0018	0.5134
HOOK3	1	-0.0038	0.5079

To gain first insights into the pathways affected by miR-3151, we performed a pathway analysis of the up- and downregulated genes (n=58) using the Ingenuity platform. The top scoring molecular and cellular functions affected by miR-3151 were Cell Death and Survival, Cellular Growth and Proliferation, and Cell Cycle (Table 2).

Table 2. ***Molecular and Cellular Functions predicted to be influenced by miR-3151 (Ingenuity).*** Listed are the top ranking components of the Global Canonical Pathway Category (GCP) after pathway analysis (www.ingenuity.com, pathway names and involved molecules defined by Ingenuity) of the miR-3151 associated gene expression signature. The *P*-value associated with a pathway in Global Canonical Pathways (GCP) is a measure of the likelihood that the association between a set of focus genes in the analyzed experiment and a given pathway is due to random chance. The *P*-value identifies statistically significant over-representation of focus genes in a given process. It is automatically calculated by the ingenuity.com program using the right-tailed Fisher Exact Test.

Pathway name (Ingenuity)	***P*-value**	**Number of molecules**
Cell Death and Survival	4.12^{-122}-1.38^{-21}	241
Cellular Growth and Proliferation	1.85^{-85}-1.2^{-21}	236
Cell Cycle	1.42^{-75}-9.3^{-22}	152
Gene Expression	1.04^{-72}-5.43^{-32}	176
Cellular Development	1.65^{-69}-1.2^{-21}	219

To distinguish between nuclear (transcriptionally active) TP53 and cytoplasmic TP53, we used cellular fractionation of Mel-39 cells after either stable introduction or knock-down of miR-3151 compared to scramble control. AntagomiR-3151 led to a preferred nuclear localization of TP53 (Figure 7A). We used confocal microscopy to validate the changes in the cellular localization caused by antagomiR-3151 in A375 and Mel-39 cells. The knock-down of miR-3151 dramatically favored the nuclear localization of TP53, thereby suggesting an increased transcriptional potential of the tumor suppressor gene caused by antagomiR-3151 (Figure 7B).

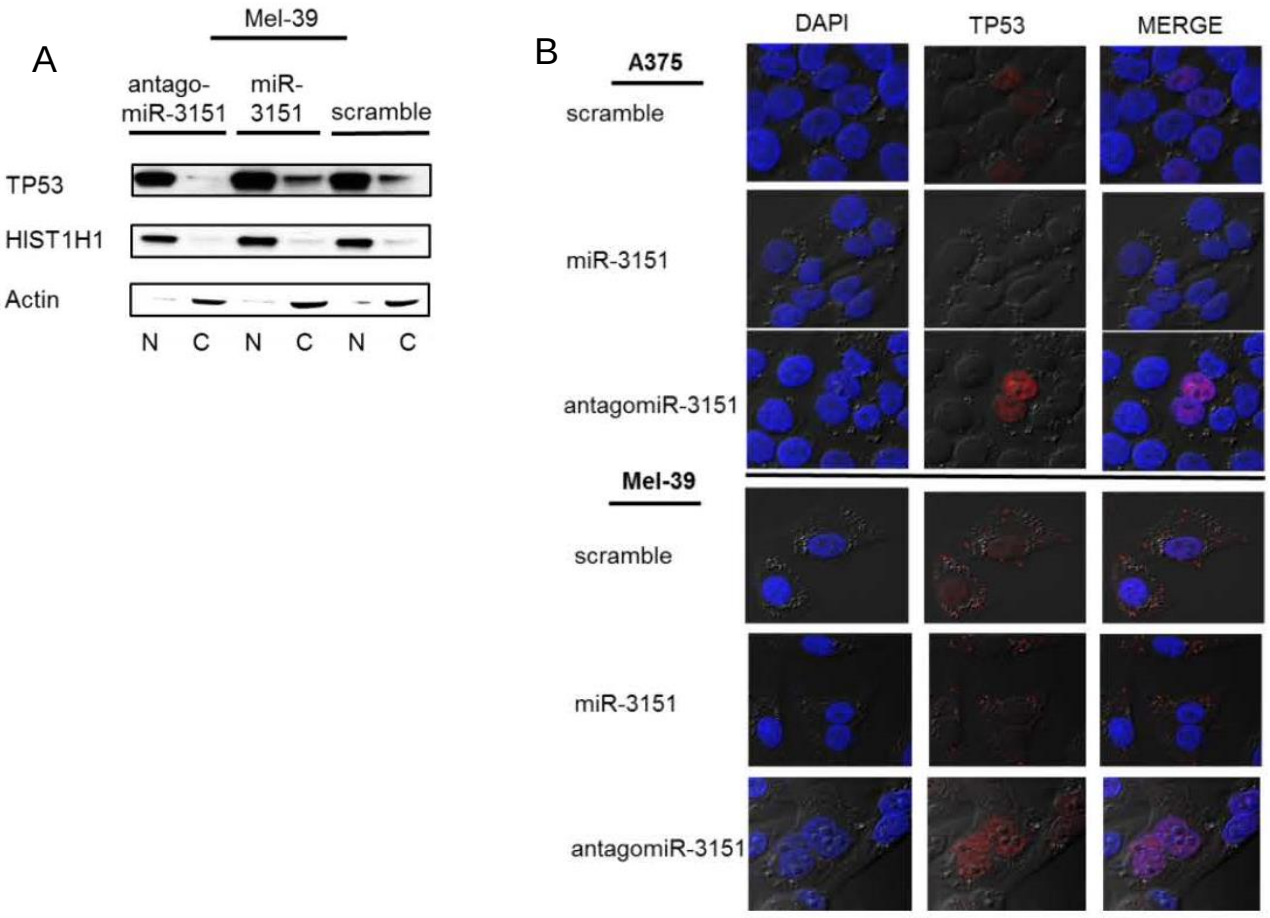


Figure 7. ***Cellular localization of TP53.*** **A.** Nuclear fractionation of Mel-39 cells after manipulation of miR-3151 expression to determine the favored cellular localization of TP53.**B.** Confocal imaging of TP53's cellular localization in A375 and Mel-39 cells after manipulation of miR-3151 expression.

Targeted knock-down of miR-3151 increases apoptosis and reduces cell viability of MM cells.

As our pilot experiments suggested an increase in caspase-3/7 activity caused by antagomiR-3151[30], we aimed to gain further evidence for a possible growth-limiting effect on MM cells, which may in part be mediated by the increased expression of *TP53*. We stably infected Mel-39 and A375 cells with miR-3151, antagomiR-3151 or scramble control, and determined the cellular proliferation rate with chemiluminescent TiterGlo assays. While increasing the abundance of miR-3151 increased the proliferation of both cell lines compared to scramble control, antagomiR-3151 led to reduced proliferation (Figure 8).

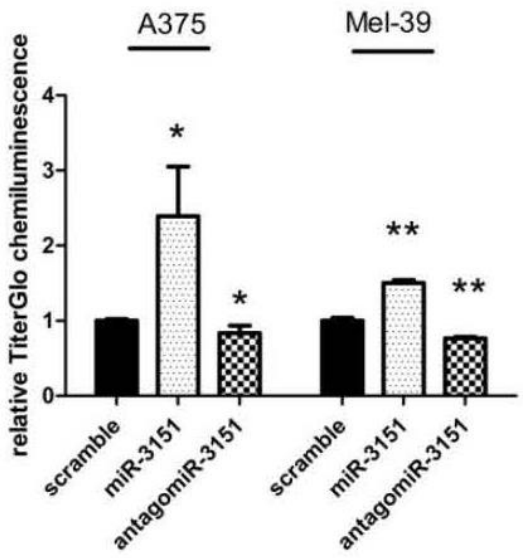


Figure 8. ***Proliferation rates in response to miR-3151.*** Cell viability of A375 and Mel-39 cells after forced miR-3151 expression or knock-down in chemiluminescent assays. 3 experiments, bars, mean ± SD; *, $P<0.05$, **, $P<0.005$, two-tailed *t* test.

The apparent decrease in growth by antagomiR-3151 could result from a decrease in the rate of cell division, an increase in apoptosis, or a combination of the two. To test whether the reduced TiterGlo activity is solely due to a lowered rate of cell division, or additionally potentiated by an increase in cell death and apoptosis, we performed a flow cytometric determination of Annexin-

V and Annexin-PI in antagomiR-3151 infected Mel-39 cells. Indeed, knock-down of miR-3151 increased the percentage of apoptotic cells (Figure 9) and also reduced the percentage of cells in both S-phase and G2-phase. Additionally, we used confocal microscopy to compare the expression and cellular localization of caspase-3, a major determinant of apoptotic activity. In line with the previous experiments, miR-3151 reduced caspase-3 expression, while antagomiR-3151 increased caspase-3 expression (Figure 10).

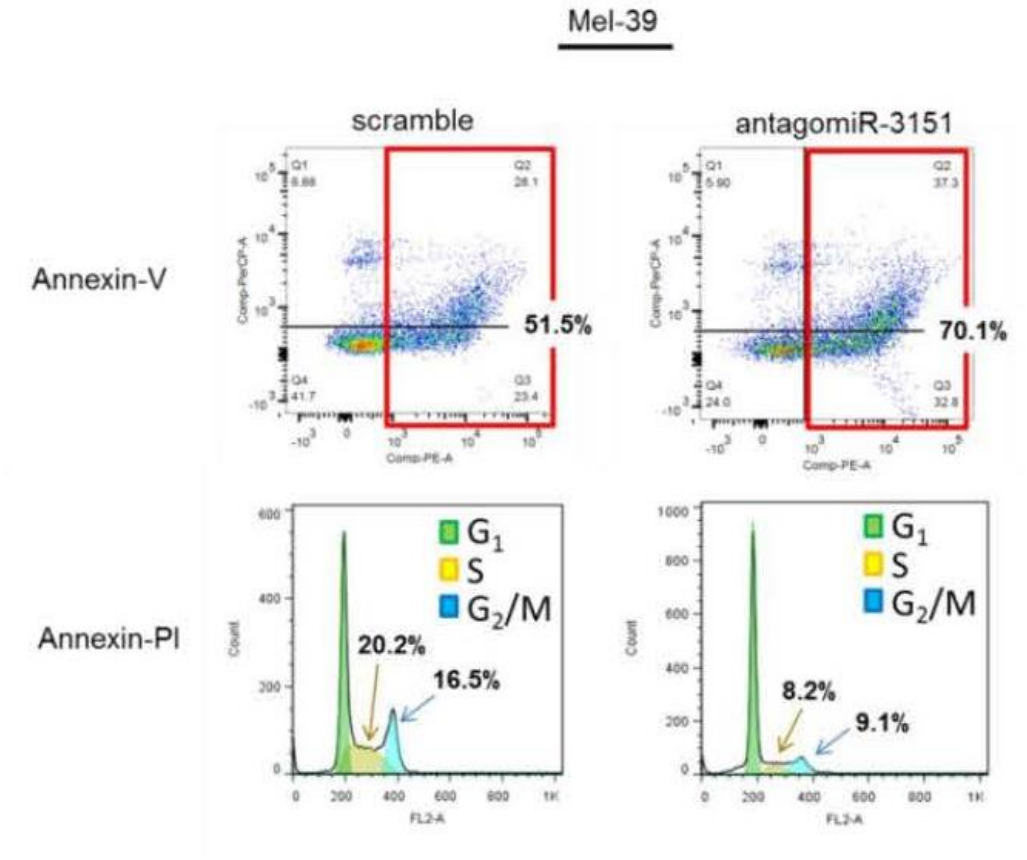


Figure 9. ***Changes in the cell cycle.*** Examples of changes in apoptosis and cell cycle in Mel-39 cells after miR-3151 knock-down. In the above two graphs, the red boxes indicate apoptotic cells or cells undergoing apoptosis. In the lower two graphs, the peak highlighted in green represents cells in the G_1 phase, the yellow peak is cells in S phase, and the blue peak is cells in G_2 and M phases.

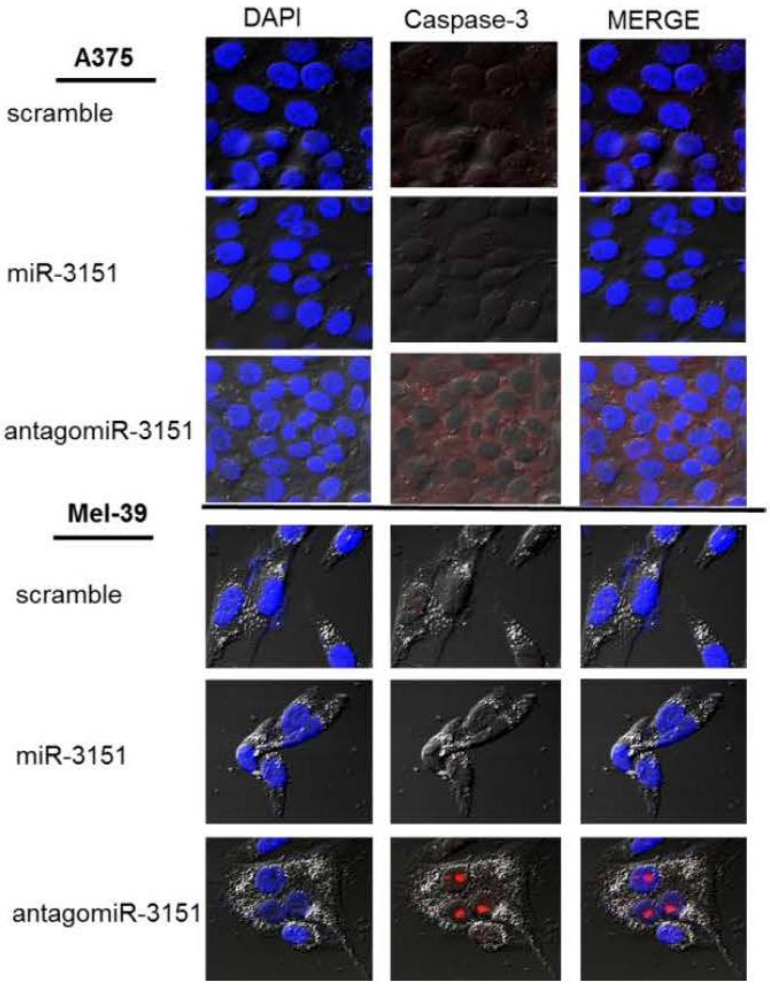


Figure 10. ***Caspase-3 cellular localization.*** Confocal imaging of caspase-3's expression and cellular localization in A375 and Mel-39 cells after manipulation of miR-3151 expression.

miR-3151 expression can be increased by BRAF mutations and the SP1/NF-κB transactivating complex.

As malignant melanoma samples with an activating BRAF mutation have a 5-fold higher expression of miR-3151 compared to BRAF wildtype (BRAFwt) samples[30], we wanted to elucidate whether miR-3151 may be a direct downstream effector of aberrantly activated BRAF and contribute to the increased disease aggressiveness associated with BRAF mutations

(BRAFmut). If miR-3151 is downstream of BRAF, an introduction of the mutation into BRAFwt cells should increase miR-3151 expression and consequently lead to a further reduction of TP53, while knock-down of BRAFmut should reduce miR-3151's abundance and elevate TP53 expression. In line with our hypothesis, transfection of MeWo cells (BRAFwt) with a BRAFmut expression construct increased miR-3151 expression and reduced TP53 expression (Figure 11A). siRNA-mediated knock-down of BRAFmut in A375 and Mel-39 cells reduced miR-3151 expression and increased TP53 at both the mRNA and protein levels (Figure 11B and 11C).

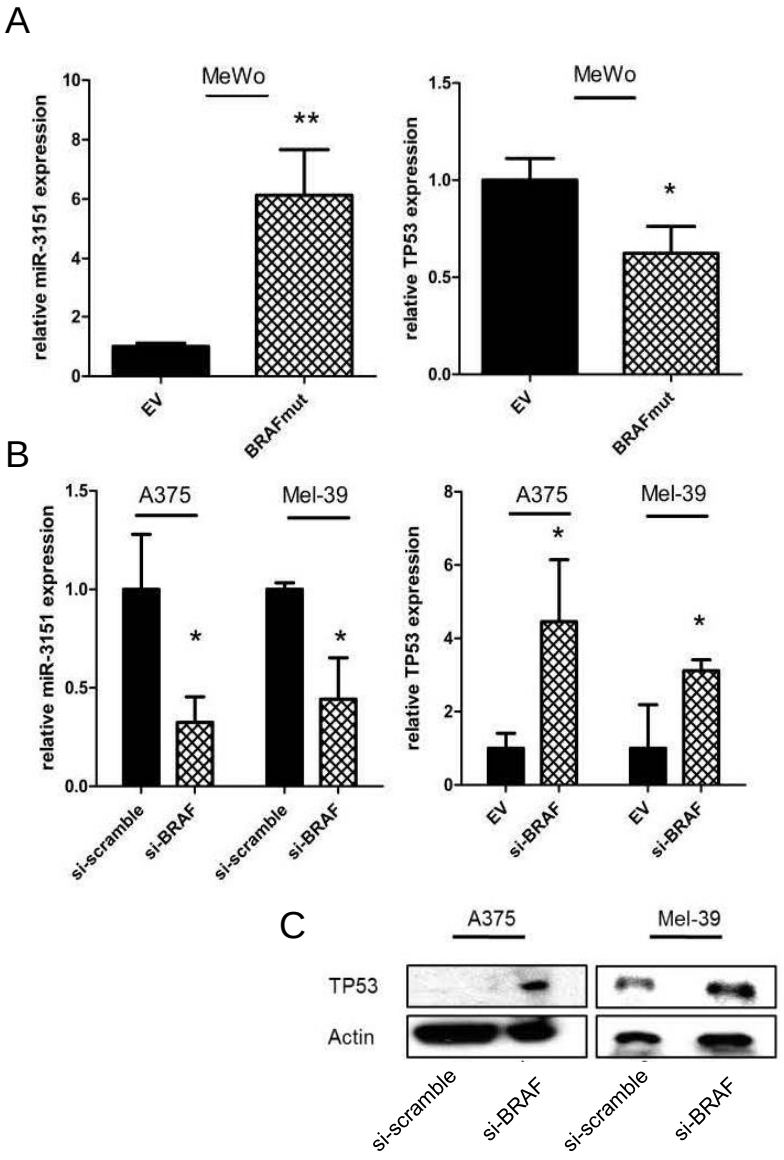


Figure 11. ***Effects of BRAFmut on miR-3151 and TP53.*** **A.** Effects of BRAFmut on miR-3151 and *TP53* expression in BRAFwt MeWo cells. 3 experiments, bars, mean ± SD; *, $P<0.05$, two-tailed *t* test. **B.** Effects of BRAF knock-down (si-BRAF) on miR-3151 and *TP53* expression in BRAFmut A375 and Mel-39 cells. 3 experiments, bars, mean ± SD; *, $P<0.05$,**, $P<0.005$, two-tailed *t* test. **C.** Example of miR-3151's effects on TP53 protein expression.

In AML, miR-3151 can be regulated by the SP1 transcription factor/ nuclear factor kappa-B (SP1/NF-κB) transactivating complex[30], which binds to the regulatory region of miR-3151 (TSS-3151). To test whether this activation also regulates miR-3151 in MM, we transfected A375, Mel-39 and MeWo cells with expression constructs for SP1 or NF-κB (p65). While A375 cells showed a greater response to SP1, Mel-39 cells reacted preferentially to transfection with NF-κB (Figure 12A). Finally, electrophoretic mobility shift assays with nuclear extracts harvested from both A375 and Mel-39 cells indicated a direct interaction of SP1/NF-κB with TSS-3151 (Figure 12B). Thus, aberrant expression of miR-3151 in melanoma seems to be a combined effect of BRAFmut and transcriptional activation by the SP1/NF-κB transactivating complex.

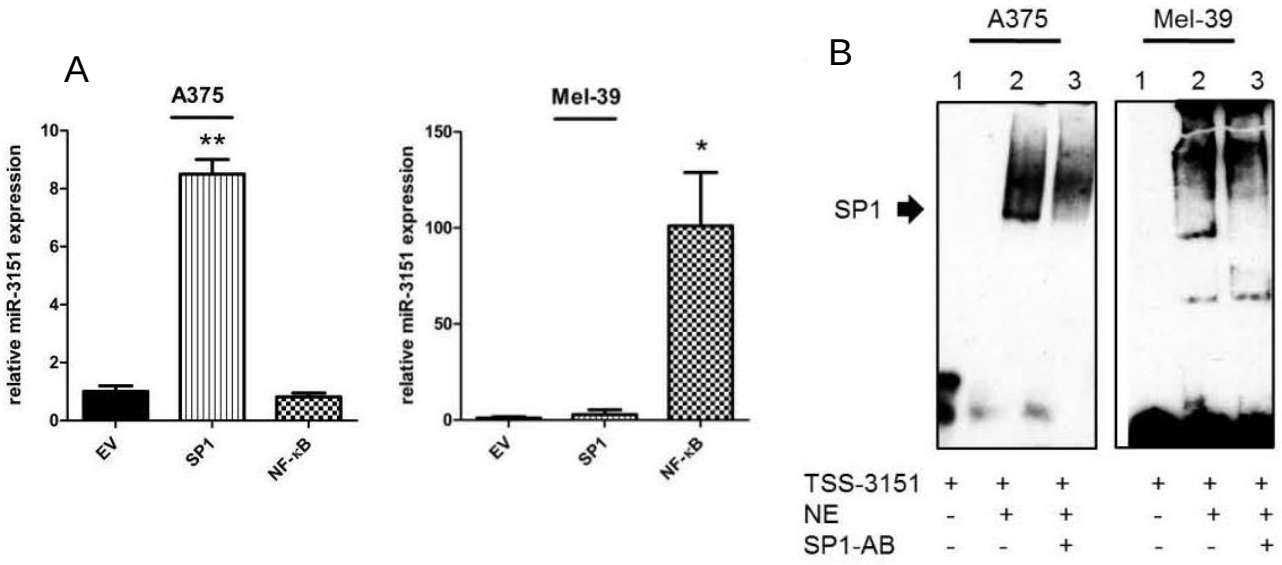


Figure 12. ***SP1/ NF-κB transactivating complex activates miR-3151 expression.*** **A.** Effects of SP1 and NF-κB on miR-3151 expression in A375 and Mel-39 cells. 3 experiments, bars, mean ± SD; *, $P<0.05$,**, $P<0.005$, two-tailed *t* test. **B.** Visualization of SP1's binding to miR-3151's transcription start site (TSS-3151) using EMSA. Nuclear extracts (NE) used from A375 and Mel-39 cells, shifting performed with SP1-antibody (SP1-AB).

The BRAF-miR-3151-TP53 axis is also present in papillary thyroid cancer.

In addition to MM, *BRAF* mutations are frequently found in papillary thyroid cancer (PTC)[32] and are associated with a more aggressive disease phenotype[33]. To test whether the newly identified axis between *BRAF* mutations, miR-3151 expression and reduced TP53 levels is also present in PTC, we first determined the BRAF mutation status and the endogenous miR-3151 expression in a cohort of 16 PTC tumor samples. As seen in MM, patients with *BRAF*mut tumors had higher endogenous miR-3151 expression (Figure 13A). In addition, patients with higher miR-3151 expression had lower endogenous *TP53* expression levels (Figure 13B).

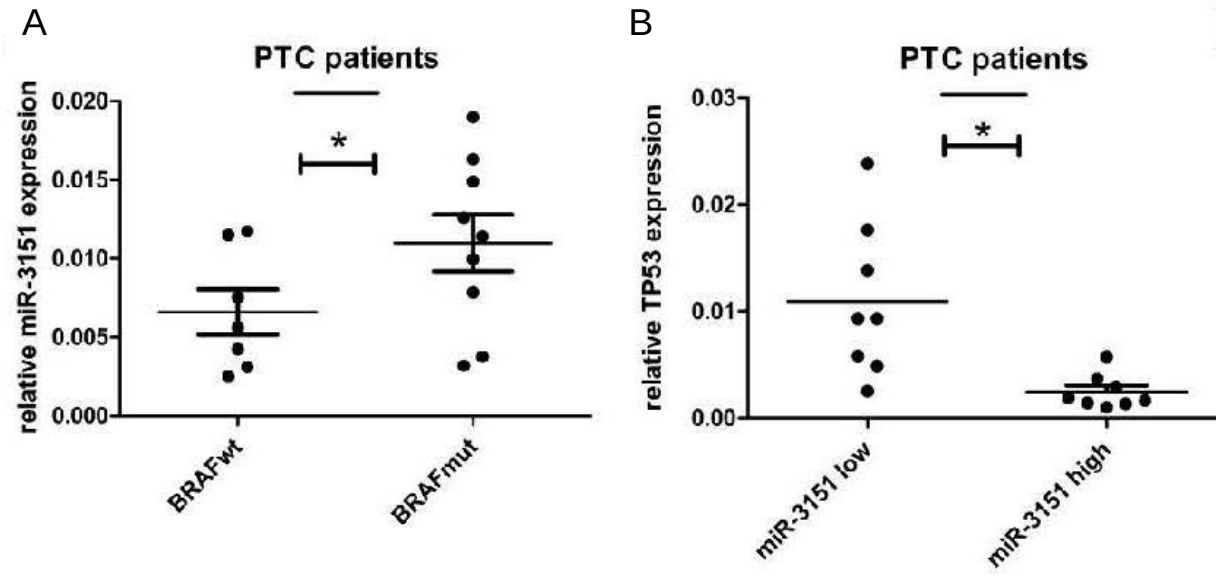
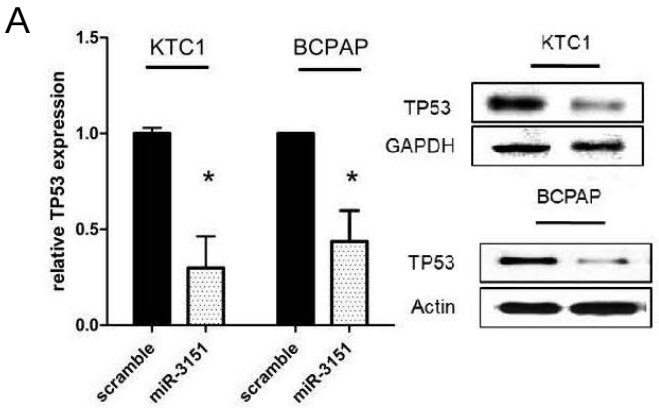


Figure 13. *miR-3151 in PTC.* **A**. Endogenous miR-3151 expression levels in tumor samples of *BRAF*wt and *BRAF*mut PTC patients. *, $P<0.05$, two-tailed *t* test. **B.** Endogenous *TP53* expression of low miR-3151 and high miR-3151 expressing PTC patients. *, $P<0.05$, two-tailed *t* test.

To validate that miR-3151 affects *TP53* expression, we stably introduced miR-3151 into two

PTC cell lines (KTC1 and BCPAP). Forced miR-3151 expression reduced TP53 at both the mRNA and protein levels (Figure 14A). Confocal microscopy of the cell lines showed a reduction of TP53 messenger expression and also a reduced nuclear localization of the protein, while antagomiR-3151 strongly enhanced the expression and nuclear localization of TP53 (Figure 10B). The changes in TP53 expression also changed the expression levels of caspase-3 indicating that knock-down of miR-3151 enhances cell death also in PTC (Figure 14B).



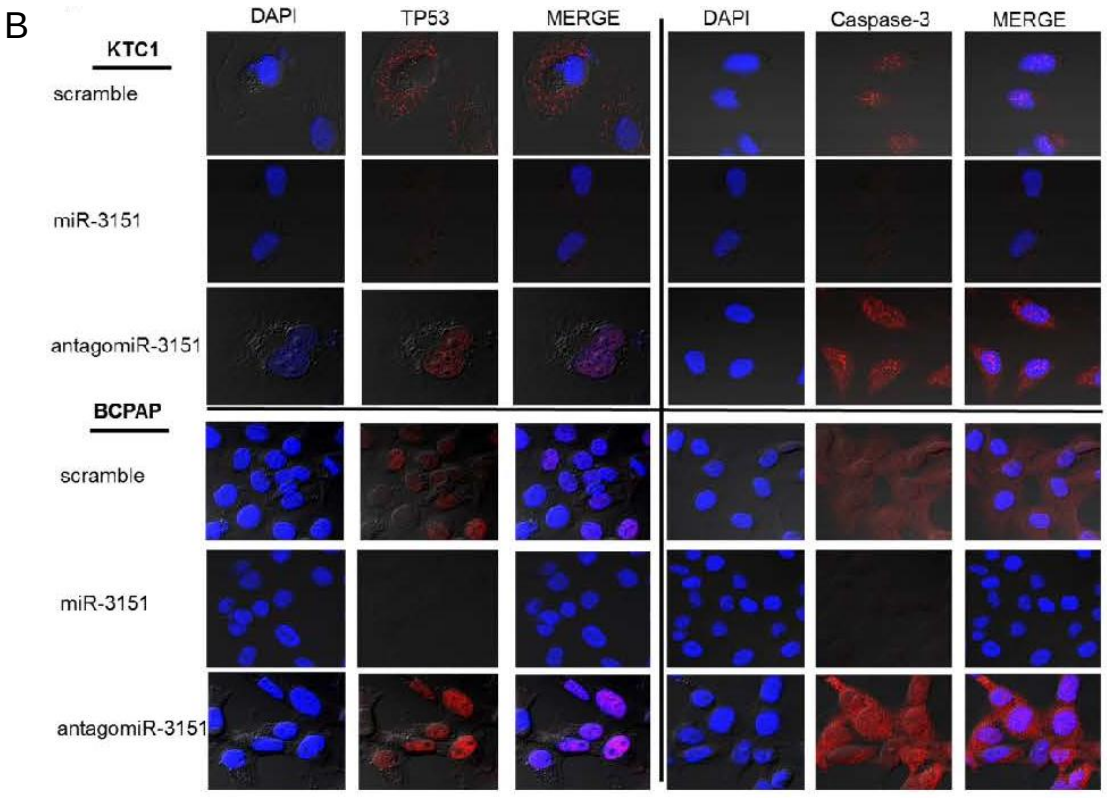


Figure 14. ***miR-3151's effects on TP53 and Caspase-3 in PTC.*** **A.** Effects of forced miR-3151 expression on TP53 mRNA and protein levels in KTC1 and BCPAP cells. 3 experiments, bars, mean ± SD; *, $P<0.05$, two-tailed *t* test. **B.** Confocal imaging of TP53's and caspase-3's expression and cellular localization in A375 and Mel-39 cells after manipulation of miR-3151 expression.

This was further supported by TiterGlo assays, which showed decreased viability in cells infected with antagomiR-3151 (Figure 15). Finally, the effects of BRAFmut knock-down on miR-3151 expression (Figure 16) and the transcriptional activation of miR-3151 by the SP1/NF-κB complex were also validated in PTC cells (Figure 17).

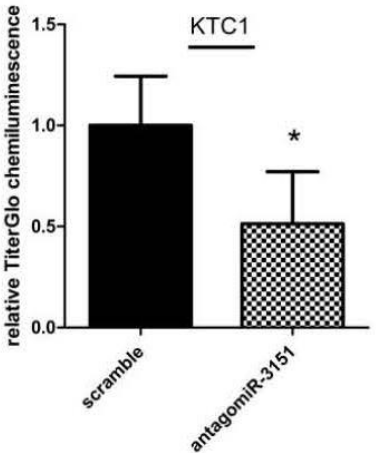


Figure 15. ***miR-3151 effects on cell viability in PTC.*** Cell viability of KTC1 cells after knock-down of miR-3151. 3 experiments, bars, mean ± SD; *****, $P<0.05$, two-tailed *t* test.

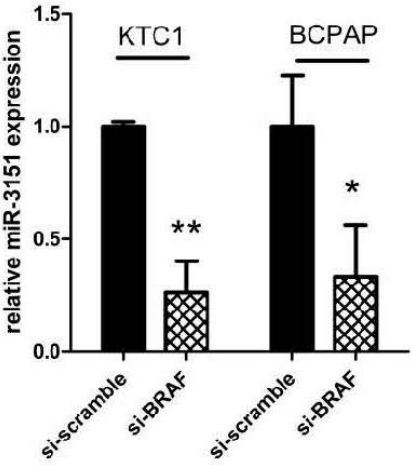


Figure 16. ***BRAFmut effects miR-3151 in PTC.*** Effects of BRAF knock-down (si-BRAF) on miR-3151 expression and *TP53* expression in KTC1 and BCPAP cells. 3 experiments, bars, mean ± SD; *****, $P<0.05$,******, $P<0.005$, two-tailed *t* test.

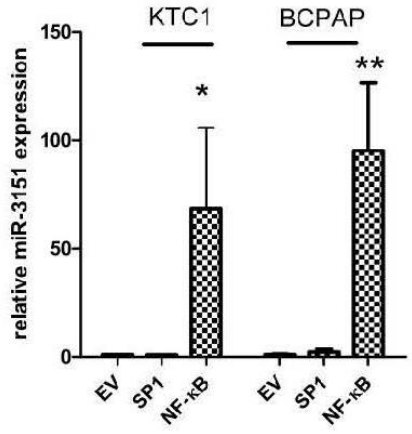


Figure 17. ***SP1/ NF-κB activation of miR-3151 in PTC.*** Effects of SP1 and NF-κB on miR-3151 expression in KTC1 and BCPAP cells. 3 experiments, bars, mean ± SD; *****, $P<0.05$,******, $P<0.005$, two-tailed *t* test.

Knock-down of miR-3151 and BRAFmut in MM and PTC cells increases sensitivity to vemurafenib treatment.

Even though we identified miR-3151 as a new downstream effector of BRAF, it is only one of many known BRAF targets. Perhaps the most important known genes affected by aberrantly activated BRAF are the *mitogen-activated protein kinase kinase 7* (MEK) and the *mitogen-activated protein kinase 1* (ERK), which facilitate signal transduction and ultimately lead to increased gene transcription and translation[34]. Of note, above we demonstrated that miR-3151 is not only activated by BRAF, but also by the SP1/NF-κB transactivation complex. Thus, to most effectively disrupt both pathways, simultaneous inhibition of BRAFmut (to stop activation of the MEK/ERK cascade and also reduce miR-3151 expression) and targeting of miR-3151 (to stop its activation by SP1/NF-κB), either by proteasome inhibitors to limit SP1/NF-κB's binding activity, or by direct miR-3151 inhibition with antagomiR-3151, may be an interesting combined treatment approach (Figure 18).

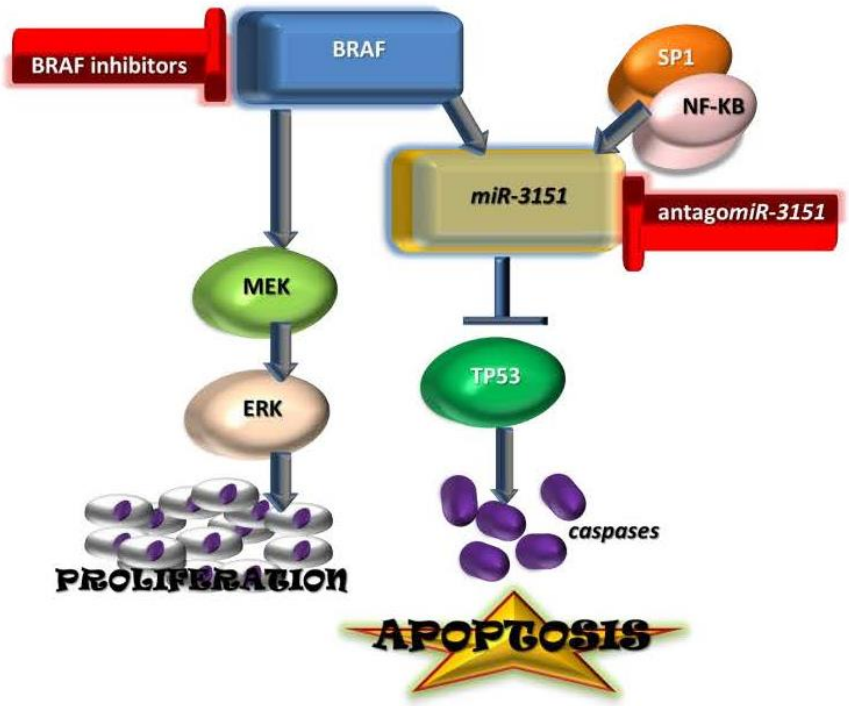


Figure 18. ***Schematic depiction of the proposed BRAF-miR-3151-TP53 axis.*** Simultaneous inhibition of BRAF (via BRAF inhibitors) and miR-3151 (shown here through antagomiR-3151) could be a possible treatment method.

As a proof-of-principle, we first tested the effects of the BRAF inhibitor vemurafenib on miR-3151 expression. Indeed, treatment of BRAFmut Mel-39 (MM) and KTC1 cells (PTC) led to a modest reduction of miR-3151 expression (Figure 19).

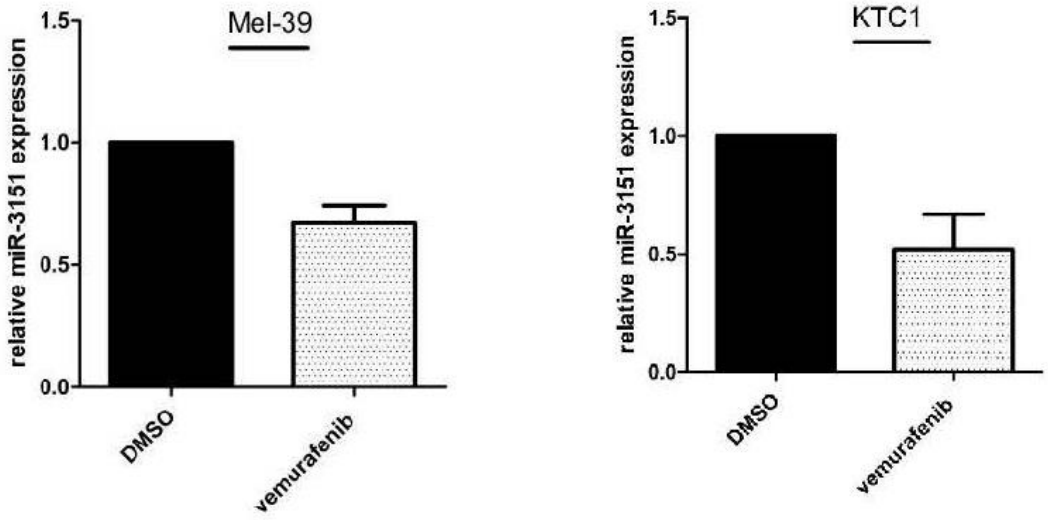


Figure 19. ***Vemurafenib treatment of MM and PTC cells.*** Effects of vemurafenib treatment on miR-3151 expression in Mel-39 (1.0 μM vemurafenib) and KTC1 cells (1.4 μM vemurafenib) compared to vehicle. 3 experiments, bars, mean ± SD.

Next, we used stably miR-3151 and antagomiR-3151 expressing Mel-39 and KTC1 cells for vemurafenib treatment and compared their response to cells infected with scramble control. While treatment with 1.0 μM (Mel-39) and 1.4 μM (KTC1) vemurafenib had no effect on cell proliferation in miR-3151 infected cells, it reduced the growth in scramble-infected cells and killed all cells with antagomiR-3151 (Figure 20). Finally, we created a vemurafenib-resistant A375 cell line and compared the endogenous miR-3151 expression level to the parental (non-resistant) A375 cells. The vemurafenib-resistant A375 cells had a 35-fold higher endogenous miR-3151 expression (Figure 21). Taken together, these pilot experiments may encourage *in vivo* tests of this combined targeted treatment approach.

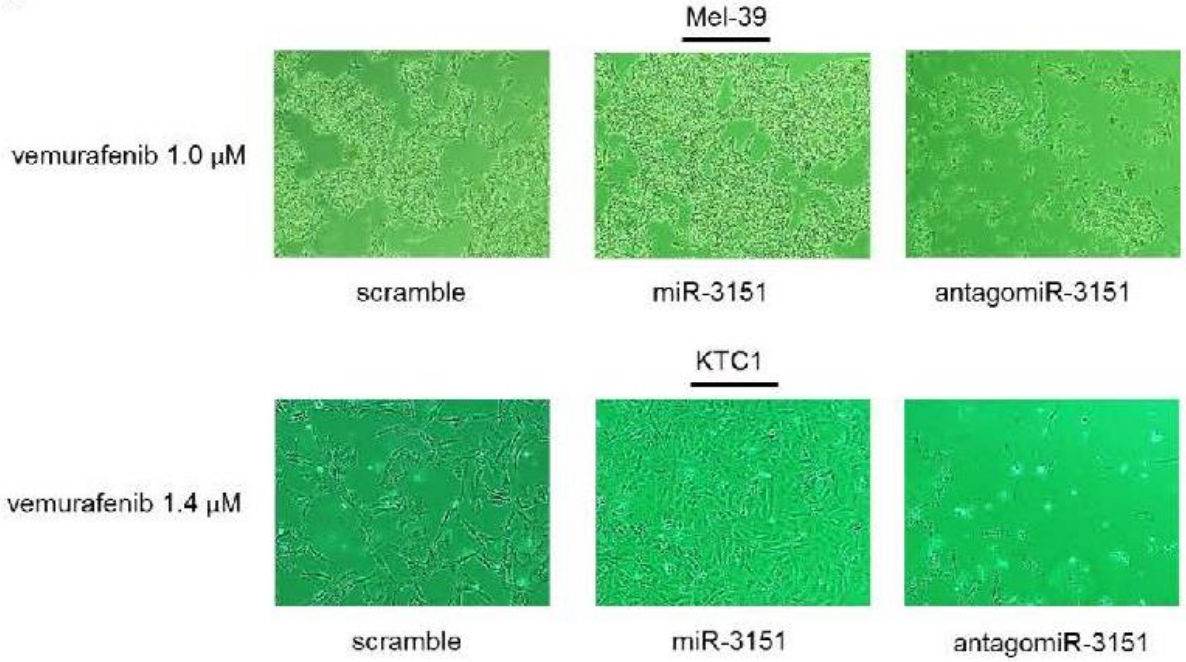


Figure 20. ***Images after vemurafenib treatment.*** Depiction of the differential effect of vemurafenib treatment on Mel-39 and KTC1 cells after forced miR-3151 expression or knock-down.

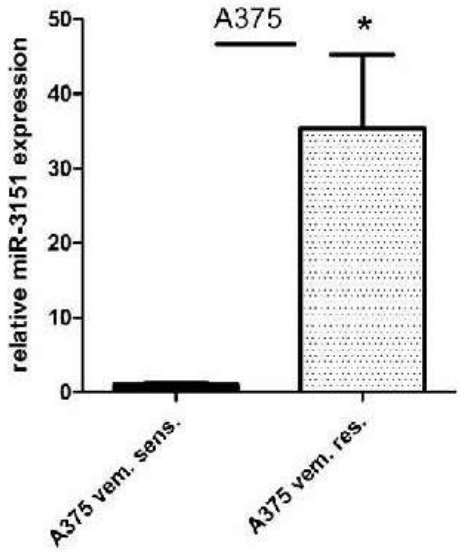


Figure 21. ***Effect of vemurafenib resistance on miR-3151.*** Changes in miR-3151 expression after development of vemurafenib resistance (A375 vem. res.) compared to the parental cell line (A375 vem. sens.). Resistant A375 cells are long-term cultured in 2 µM vemurafenib. 3 experiments, bars, mean ± SD; *, $P<0.05$, two-tailed *t* test.

Discussion

MM is the most deadly skin cancer, accounting for 75% of all skin cancer deaths with numbers continuously increasing[1]. When diagnosed at an advanced stage (stage IV), the 5-year survival rates of the patients are only ~15%[1], underlining the urgent need for better treatment options for advanced stage patients. *BRAF* mutations are the most frequent mutations in MM and are associated with increased disease aggressiveness[6]. Therefore, targeting BRAF has become one of the most promising treatment options in BRAF mutated MM patients[8]. Still, many questions regarding the downstream signaling of mutated BRAF remain unanswered.

As a second crucial event, the tumor suppressor *TP53* is downregulated in almost all MM**,** but the reasons are not fully understood[9]. Restoration of *TP53* activity represents an important treatment strategy in MM[10]. The *BRAF* mutation and repression of *TP53* are important events in melanoma progression. In fact, disruption of the *TP53* pathway via short hairpin RNA in benign nevi with *BRAF* mutations may promote malignant transformation of the cells[11]. Thus, the identification of a causal link between these two important players (BRAF and TP53) would increase our understanding of MM pathophysiology.

Vemurafenib is an orally administered**,** small molecule selective BRAF inhibitor approved by the FDA for the treatment of unresectable or metastatic MM with presence of the BRAF V600E mutation[35]. In an international multicenter trial (NCT01006980), treatment with vemurafenib was superior to treatment with chemotherapy (dacarbazine)[36]. However, not all patients benefited from treatment with vemurafenib[36, 37].

Deregulation of specific miRs has been implicated in the disease initiation and progression of virtually every human cancer[38, 39], including MM[40]. Specific targeting of aberrantly activated miRs, either indirectly via blockage of their upstream activation or direct via antagomiRs,

represents a promising new therapeutic approach[41, 42].

We have shown that miR-3151 is upregulated in *BRAF*mut MM and have provided the first evidence that miR-3151 leads to reduced MM cell growth by direct downregulation of its target *TP53*. We propose that miR-3151 may be a crucial link between *BRAF* mutations and the observed downregulation of *TP53*.

Additional upregulation of miR-3151 by BRAF-independent mechanisms (SP1/NF-κB transactivating complex) may explain why some patients show tumor progression under vemurafenib treatment, and may be an alert to combine vemurafenib with other chemotherapeutics.

Determination of miR-3151 and *TP53* expression in *BRAF*mut MM tumors during vemurafenib therapy will provide insight into the predictive value of miR-3151 expression. Connecting *BRAF* mutations with *TP53* downregulation via increased expression of miR-3151 is a testable approach in MM research. Further elucidation of the mechanism by which *BRAF*mut upregulates miR-3151 will increase our understanding of the BRAF mutant downstream biology and enhance our options for therapeutic interventions.

Materials and Methods

Patient samples

PTC samples (n=16) were obtained from the Human Cancer Genetics Tissue Bank at OSU. All patients provided written informed consent according to the Declaration of Helsinki to store and use their tissue for discovery studies according to OSU institutional guidelines under protocols approved by the OSU Institutional Review Board. Total RNA samples from MM patients (n=21) were purchased from Asterand.

Tissue culture experiments

For studying miR-3151 in MM A375, Mel-39 and MeWo cells were provided by the laboratory of W.E. Carson. Cells were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic-Antimycotic (Gibco). For studying miR-3151 in PTC, KTC1 and BCPAP cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic-Antimycotic (Gibco).

To create vemurafenib resistant A375 cells, the cells were cultured in increasing concentrations of vemurafenib (0-10 μM) over a period of 8 weeks with 2-fold dosage increases once a week. After confirmation of resistance using MTT assays, the cells were cultured in 2 μM vemurafenib.

Overexpression of miR-3151 and miR-3151 knockdown

For stable expression, the stem loop of miR-3151 with 200 bp flanking sequence was cloned into an HIV-based lentiviral dual promoter vector as previously described (pCDH-CMV-MCS-EF1-copGFP+Puro cDNA; System Biosciences). For targeted knock down of miR-3151, a custom-made antago*miR-3151* was purchased from System Biosciences. As a control, lentiviral scramble miR was used according to the manufacturer's instructions (miRZiP000, System Biosciences).

Lentiviral construct (4500 µg) was transfected into 293TN cells using 45 µg pPACKH1 and 55 µl PureFection (System Biosciences). After 48h and 72h the supernatant containing the pseudoviral particles was collected and the virus precipitated overnight at 4°C using 5 ml PEG-IT virus precipitation solution (System Biosciences). We used 200 µl Phosphate Buffered Saline and 25 µM Hepes Buffer for resuspension of the pelleted virus. We infected 200,000 cells/ml in triplicate with 20 IU virus, using 5 µl Transdux Infection Reagent (System Biosciences).

cDNA synthesis and miR-3151 mRNA analysis

To check for successful overexpression of *miR-3151* and to analyze the effect of forced *miR-3151* expression on the predicted target genes, RNA from 1 million cells was harvested on day 14 after infection and reverse transcribed to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Corporation/Applied Biosystems) or the Superscript III First-Strand cDNA Synthesis Kit *(*Life Technologies Corporation/Invitrogen*).* Both kits were used according to the manufacturer's instructions. miR-3151 abundance was determined by qRT-PCR as previously described (21). Simultaneously protein (from 4 million cells) was harvested and used for Western blotting.

Transient overexpression of BRAFwt, BRAFmut, SP1 and NF-κB

For transient overexpression, 3 µg of the overexpression constructs of *BRAF*wt, *BRAF*mut, *SP1, NF-κB* and (all cloned in p*IRES2-EGFP* vector, Clontech) or the p65 subunit of NF-κB (cloned as pCMV-p65) were transfected in triplicate into 3 million Mel-39 and A375 cells using Purefection transfection reagent according to the manufacturer's instructions (System Biosciences).

TruSeq targeted RNA analysis

A customized Add-on panel comprised of 361 genes using the backbone of the Illumina

apoptosis panel and the Illumina stem cell panel was designed using DesignStudio (Illumina) for the TruSeq Targeted RNA expression analysis. Library preparations using 100 ng total RNA (miR-3151+scramble: harvest 3h post transfection, antagomiR-3151/scramble: harvest 24h post transfection) and the Miseq run were performed according to the manufacturer's instructions. MiSeqReporter software was used to estimate target hits for each transcript after aligning reads against references specified by Targeted Oligo Pool, using banded Smith-Waterman alignment. The raw count data was then normalized using the R library DESeq (V 1.14.0), built based on negative binomial distribution, with variance and mean linked by local regression (20). Percent relative changes of mRNA expression of miR-3151 and antagomiR-3151 compared to scramble were estimated.

TiterGlo assays

Cell viability changes in Mel-39, A375 and KTC1 cell lines infected with lentiviral miR-3151, antagomiR-3151, or scramble control were analyzed using the Titer Glo assay (Promega) 72h after Puromycin selection using 20,000 cells in duplicate of three biological replicates according to the manufacturer's instructions.

Western blotting assays

Western blotting was performed according to standard procedures. Antibodies used were p53 (#sc-126, Santa Cruz), Caspase-3 (#9665S, Cell Signaling), Actin (#sc-1616, Santa Cruz).

Confocal microscopy

Confocal staining was performed by standard procedures using the following antibodies: p53 Ab-2 (Oncogene Research Products OP09), Caspase-3 (Cell Signaling #9665), Alexa Flour 546 donkey anti-rabbit (Life Technologies A10040), and Alexa Flour 647 goat anti-mouse (Life Technologies A21240). Confocal micrographs were taken using the FV1000 Confocal Laser

Scanning Microscope (Olympus) with a UPLFLN 40x Oil, N.A. 1.3 lens.

Electrophoretic mobility shift assay

Nuclear proteins were extracted from Mel-39 and A375 cells using the Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. All oligonucleotide sequences used for the EMSA analysis are: TSS3151 SP1 Forward EMSA Biotin 5'-/5Biosg/GCAGTGGGGTGGGGTTTGGA-3 and TSS3151 SP1 Reverse EMSA Biotin 5'-/5Biosg/ TCCAAA CCCCACCCCACTGC-3. For EMSA, the Thermo Scientific LightShift Chemiluminescent EMSA Kit (Pierce/Thermo Fisher Scientific) was used according to the manufacturer's instructions. Antibodies used were NF-κB p65 (#sc-71677, Santa Cruz), SP1 (#sc-59, Santa Cruz).

Statistical methods

Data were represented as mean ±standard deviation (s.d.) of at least three independent experiments, unless otherwise indicated, and analyzed by the two-tailed or one-tailed Student's *t*-test. The means and s.d. were calculated and displayed in bar graphs as the height and the corresponding error bar, respectively. A $P < 0.05$ was considered statistically significant.

References

1) "Surveillance, Epidemiology, and End Results ProgramTurning Cancer Data Into Discovery." *Melanoma of the Skin*. National Cancer Institute, n.d. Web. 10 Mar. 2015.

2) "Skin Cancer Foundation." Melanoma. Skin Cancer Foundation, n.d. Web. 11 Feb. 2015.

3) "New York Thyroid Center." Papillary Thyroid Cancer. Columbia University Department of Surgery, n.d. Web. 12 Mar. 2015.

4) "BRAF Gene." *Genetics Home Reference*. N.p., Dec. 2012. Web. 12 Mar. 2015.

5) Groussin, Lionel, and James A. Fagin. "Significance of BRAF Mutations in Papillary Thyroid Carcinoma: Prognostic and Therapeutic Implications." Nat Clin Pract Endocrinol Metab 2.4 (2006): 180-81. Medscape. Nature Publishing Group. Web. 12 Mar. 2015.

6) Davies, H, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–954.

7) Melck, Adrienne L., Linwah Yip, and Sally E. Carty. "The Utility of BRAF Testing in the Management of Papillary Thyroid Cancer." The Oncologist 2010;15(12): 1285-293.

8) Lito, P, Rosen, N, Solit, DB. Tumor adaptation and resistance to RAF inhibitors. Nat Med 2013; 19:1401-9.

9) Houben, R, Hesbacher S, Schmid CP, Kauczok CS, Flohr U, Haferkamp S, et. al. High-level expression of wild-type p53 in melanoma cells is frequently associated with inactivity in p53 reporter gene assays. PLoS ONE 2011;6: 1-7.

10) Box, NF, Vukmer, TO and Terzian, T, Targeting p53 in melanoma. Pigment Cell & Melanoma Research 2013;27:8-10.

11) Yu H, McDaid R, Lee J, Possik P, Li L, Kumar SM, et al. The role of BRAF mutation and

p53 inactivation during transformation of a subpopulation of primary human melanocytes. Am J Path 2009;174:2367-2377.

12) Malaguarnera, R., V. Vella, R. Vigneri, and F. Frasca. "P53 Family Proteins in Thyroid Cancer." Endocrine-Related Cancer 14 (2007): 43-60. Web.

13) "Tumor Grade." *National Cancer Institute*. N.p., 3 May 2013. Web. 12 Mar. 2015.

14) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116:281-297.

15) Calin GA, Croce CM: MicroRNA signatures in human cancers. Nat Rev Cancer 6:857 866, 2006

16) Calin GA, Croce CM: MicroRNA-cancer connection: The beginning of a new tale. Cancer Res 66:7390-7394, 2006

17) Marcucci G, Radmacher MD, Maharry K, et al. MicroRNA expression in cytogenetically normal acute myeloid leukemia. *N Engl J Med.* 2008;358(18):1919-1928.

18) Garzon R, Volinia S, Liu C-G, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood.* 2008;111(6):3183-3189.

19) Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 2004;14(10a):1902-1910.

20) Griffiths-Jones S. Annotating noncoding RNA genes. Annu Rev Genomics Hum Genet. 2007;8:279-298.

21) Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA. 2005;11:241–247

22) Kim YK, Kim VN Processing of intronic microRNAs. EMBO 2007; J 26:775–783

23) Wang X, Xuan Z, Zhao X, Li Y, Zhang MQ. High-resolution human core-promoter prediction with CoreBoost_HM. Genome Res. 2009;19:266–275

24) Corcoran DL, Pandit KV, Gordon B, Bhattacharjee A, Kaminski N, Benos PV. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. PLoS One. 2009;4(4):e5279. Epub 2009 Apr 23.

25) Monteys AM, Spengler RM, Wan J, Tecedor L, Lennox KA, Xing Y, Davidson BL. Structure and activity of putative intronic miRNA promoters.RNA. 2010 Mar;16(3):495-505. Epub 2010 Jan 14

26) Najafi-Shoushtari SH, Kristo F, Li Y, et al. MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science. 2010;328(5985):1566-1569.

27) Rayner KJ, Suárez Y, Dávalos A, Parathath S, Fitzgerald ML, Tamehiro N, Fisher EA, Moore KJ, Fernández-Hernando C., MiR-33 contributes to the regulation of cholesterol homeostasis. Science. 2010 Jun 18;328(5985):1570-3.

28) Barik S. et al. An intronic microRNA silences genes that are functionally antagonistic to its host gene. Nucleic Acids Res. 2008 Sep;36(16):5232-41

29) Veronese A, Lupini L, Consiglio J, Visone R, Ferracin M, Fornari F, Zanesi N, Alder H, D'Elia G, Gramantieri L, Bolondi L, Lanza G, Querzoli P, Angioni A, Croce CM, Negrini M. Oncogenic role of miR-483-3p at the IGF2/483 locus. Cancer Res. 2010 Apr 15;70(8):3140-9.

30) Eisfeld AK, Schwind S, Patel R, Huang X, Santhanam R, Walker CJ, et al. Intronic miR-3151 hosted by BAALC drives leukemogenesis by direct deregulation of the TP53 pathway. Science Signaling 2014;7:1-21.

31) Eisfeld AK, Marcucci G, Maharry K, Schwind S, Radmacher MD, Nicolet D, et al. miR-

3151 interplays with its host gene BAALC and independently impacts on outcome of patients with cytogenetically normal acute myeloid leukemia. Blood 2012;120:249-58.

32) Jo YS, Li S, Song JH, Kwon KH, Lee JC, Rha SY, et al. Influence of BRAF V600E mutation on expression of vascular endothelial growth factor in papillary thyroid cancer. J Clin Endocr Metab 2006; 91:3667-3670.

33) Xing M, Alzahrani AS, Carson KA, Viola D, Elisei R, Bendlova B, et al. Association between BRAF V600E mutation and mortality in patients with papillary thyroid cancer. JAMA 2013; 309:1493-1501.

34) Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006; 439:358-362.

35) "FDA Approval for Vemurafenib." *National Cancer Institute*. N.p., 3 July 2013. Web. 12 Mar. 2015.

36) Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med 2011; 364:2507-2516.

37) Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. N Engl J Med 2012; 366:707-714.

38) Lee SK, Calin GA. Non-coding RNAs and cancer: new paradigms in oncology. Discov Med 2011; 11:245-54.

39) Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol 2014; 9:287-314.

40) Mueller DW, Bosserhoff AK. The evolving concept of “melano-miRs”-microRNAs in

melanomagenesis. Pigment Cell Melanoma Res 2010; 23:620-6.

41) Monroig PD, Chen L, Zhang S, Calin GA. Small molecule compounds targeting miRNAs for cancer therapy. Adv Drug Deliv Rev 2014; pii:S0169-409X(14)00200-0.

42) Li Z, Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. Nat Rev Drug Discov 2014; 13:622-38.

43) "Apoptosis Signaling Pathway - @ a Glance." Abcam. N.p., n.d. Web. 12 Mar. 2015.

Funding: This work was supported in part by the National Cancer Institute (grants P30 CA16058, P01 CA124570), the Coleman Leukemia Research Foundation and the Pelotonia Fellowship Program.